

Effects of Tannic Acid on Cecal Volatile Fatty Acids and Susceptibility to *Salmonella typhimurium* Colonization in Broiler Chicks¹

L. F. Kubena,² J. A. Byrd, C. R. Young, and D. E. Corrier

USDA, ARS, Southern Plains Agricultural Research Center, Food and Feed Safety Research Unit,
2881 F&B Road, College Station, Texas 77845

ABSTRACT Young chickens are more susceptible to *Salmonella* colonization than older chickens that have developed resistance with age as native microflora become established. Elevated concentrations of cecal propionic acid and total volatile fatty acids (VFA) have been observed by many researchers to be indicators of establishment of anaerobic microflora and protection against *Salmonella* colonization of the ceca. Disruption of the native microflora or competitive exclusion (CE) cultures by components of diets, such as tannic acid (TA), could alter the concentrations of propionic acid and total VFA and possibly affect *Salmonella* colonization. Two experiments were conducted using day-of-hatch, mixed-sex broiler chicks to evaluate the effects of TA on cecal VFA and the susceptibility to *Salmonella* colonization. All chicks in both experiments were challenged orally with 10^4 cfu of *Salmo-*

nella typhimurium (ST) on Day 3 (Experiment 1) or Day 4 (Experiment 2). One-half of the chicks were orally gavaged on the day of hatch with a CE culture (PREEMPT™) and were fed diets containing 0, 0.75, or 1.5% TA for up to 12 d of age. Chicks were maintained in batteries in separate rooms for the experimental period. There were some alterations in concentrations of cecal propionic acid or total VFA in chicks fed diets containing 0.75 or 1.5% TA in non CE-treated chicks and in CE-treated chicks. No significant differences were observed for numbers of *Salmonella* cecal culture-positive chicks or in the numbers of ST in the cecal contents due to dietary content of TA. With minor exceptions, the chicks treated with the CE culture had higher cecal concentrations of propionic acid and were less susceptible to *Salmonella* colonization than the non CE-treated chicks. Further research is necessary to determine the biological significance of these changes.

(Key words: salmonella, broiler chicks, cecal bacteria, volatile fatty acids, tannic acid)

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INTRODUCTION

In recent years there has been increased consumer concern regarding the contamination of products with salmonellae. Poultry products are among those products frequently contaminated with various serotypes of *Salmonella* and these products are a source of human salmonellosis (Cohen and Tauxe, 1986; Izat et al., 1991). Numerous factors can affect the susceptibility of chickens to salmonellae colonization, including age, stress, general health, feed additives, the genetics of the chicken, and others (Bailey, 1988).

Newly hatched chicks are more susceptible to salmonellae colonization than older chicks that have developed resistance as native microflora become established.

This increased susceptibility to salmonellae cecal colonization has been attributed to insufficient concentration of cecal volatile fatty acids (VFA) to prevent colonization (Barnes et al., 1979). Barnes et al. (1980) reported that cecal VFA concentrations are indicators of anaerobe growth. Nisbet et al. (1994, 1996) and Corrier et al. (1995) confirmed this hypothesis and demonstrated a correlation between cecal VFA concentrations, especially propionic acid, in 3-d-old chicks with the establishment of anaerobic cecal microflora and protection against *Salmonella typhimurium* (ST) colonization.

Adequate information on the effects of specific dietary constituents on the native microflora or competitive exclusion (CE) cultures is not available. Some grain sorghum varieties available for use in poultry diets contain high levels of tannins. The use of these grain sorghums with a high tannin content in poultry diets and the addition of tannic acid (TA) to experimental diets have been

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²To whom correspondence should be addressed: kubena@usda.tamu.edu.

Abbreviation Key: BGA = brilliant green agar; CE = competitive exclusion; CEC = competitive exclusion culture; NCEC = natural competitive exclusion; ST = *Salmonella typhimurium*; TA = tannic acid; VFA = volatile fatty acid.

shown to cause reduced performance of chickens (Chang and Fuller, 1964; Vohra et al., 1966; Conner et al., 1969; Armstrong et al., 1973, 1974; Rostagno et al., 1973; Dale et al., 1980; Kubena et al., 1983). Tannins have been shown to increase the protein requirement of chicks (Rostagno et al., 1973), with methionine being specifically required by the chick to detoxify tannins (Fuller et al., 1967). Tamir and Alumot (1970) noted the formation of an insoluble tannin-protein complex in the gastrointestinal tract. Tannin-containing feedstuffs, such as grain sorghum, are routinely used in poultry diets to the extent that the level of tannins in the diet remains below what is believed to be detrimental to performance.

The purpose of the present research was to evaluate the effects of TA on cecal propionic acid, total VFA concentrations, and ST colonization of ceca in young chicks with native (unaltered) microflora and chicks administered a commercially characterized CE culture.

MATERIALS AND METHODS

Two experiments were conducted using day-of-hatch mixed-sex broiler chicks obtained from a commercial hatchery. The chicks were maintained in electrically heated batteries under continuous fluorescent lighting with feed and water provided ad libitum. The diets were formulated without added antibiotics, coccidiostats, or growth promoters and contained or exceeded the levels of nutrients recommended by the National Research Council (1994). A primary poultry isolate of ST from the National Veterinary Services Laboratory (Ames, Iowa) was selected for resistance to novobiocin and nalidixic acid in the Food and Feed Safety Laboratory, Southern Plains Agricultural Research Center, and was maintained on nutrient agar. Media used to culture the antibiotic-resistant isolate in experimental studies contained 25 μg of novobiocin and 20 μg of nalidixic acid/mL to inhibit the growth of other bacteria. Challenge inocula were prepared from an overnight culture that previously had been transferred three times in trypticase soy broth. The paper liners from the chick transport boxes were cultured successively in buffered peptone water, in selenite cystine broth, and on brilliant agar (BGA) plates as described previously (Andrews et al., 1978); plates were examined for salmonellae on an automatic colony counter.³ One or two suspect *Salmonella* colonies were confirmed by biochemical tests on triple sugar agar and lysine iron agar³ and were further identified as ST serologically using *Salmonella* O antiserum, Group 13, Factors 1, 4, 12, 15.⁴ *Salmonella* spp. were not detected in the paper liners.

In Experiments 1 and 2, 480 chicks were randomly assigned to 6 groups of 80 chicks each (4 replicates of

20 chicks each). Two hundred forty chicks were orally gavaged with a CE culture (PREEMPTTM),⁵ a commercial CE product, according to manufacturer's recommendations (CEC), and 240 were allowed to develop their microflora naturally (NCEC) under similar conditions in a separate room. The chicks were fed diets containing 0 (controls), 0.75% TA, or 1.5% TA for up to 12 d. The chicks were challenged by crop gavage at 3 d of age (Experiment 1) or 4 d of age (Experiment 2) with 10^4 cfu ST. In Experiment 1, at 3, 6, 8, and 11 d of age, 12 chicks per treatment group (four replicates of three chicks each) were killed by cervical dislocation, and cecal contents from each chick were collected aseptically. Body weights were obtained at the conclusion of Experiment 1. In Experiment 2, the same procedure was followed except sampling was performed at Days 4, 8, and 12, and body weights were not obtained at the conclusion of the experiment. The concentrations of propionic acid and total VFA (acetic, propionic, butyric, isobutyric, valeric, and isovaleric) in the cecal contents were determined by gas liquid chromatography as described by Corrier et al. (1995). On Day 11 (Experiment 1) or Day 12 (Experiment 2), 0.25-g samples of the contents of one cecum from each of the 12 chicks per treatment were serially diluted and spread-plated on BGA plates at dilutions of 1:100, 1:1000, and 1:10,000. The plates were incubated for 24 h at 37 C, and the number of cfu of ST per gram of cecal contents was determined on an automatic colony counter.⁴ Suspect *Salmonella* colonies were confirmed by biochemical tests and were further identified as ST by using serological tests described above. *Salmonella* colony plate counts were expressed as \log_{10} *Salmonella* per gram of cecal contents. Cecal contents that were *Salmonella* culture negative at the 1:100 dilution but positive after culture in selenite-cystine were arbitrarily assigned a value of 1.50 \log_{10} *Salmonella* per gram of cecal contents. Selenite-cystine cultures that were negative on BGA plates were assigned a \log_{10} *Salmonella* value of 0.

Data were subjected to ANOVA (Snedecor and Cochran, 1967) using the general linear models procedure of a statistical software (SAS Institute, 1987). Significant differences were further separated using the Fischer's protected least-significant difference (LSD) procedure (Snedecor and Cochran, 1967). Chi-squared analysis was used to determine significant differences between treatments in *Salmonella* cecal colonization rates (Luginbue and Schlotzhauer, 1987). All statements of significance are based on $P < 0.05$, unless otherwise stated.

RESULTS

Within the groups of NCEC chicks, compared with controls in Experiment 1, the only alteration in concentrations of cecal propionic acid and total VFA was a significant reduction in total VFA at 11 d in chicks fed the 1.5% diet (Table 1). The range of values for the NCEC treatments were 0.00 to 5.97 and 11.39 to 77.81 at 3 d, 0.00 to 3.36 and 15.98 to 50.19 at 6 d, 0.00 to 40.81 and 23.66 to 93.04 at 8 d, and 1.77 to 28.74 and 43.88 to 126.41

³Biotran III, New Brunswick Scientific Co., Edison, NJ 08818-4004.

⁴Difco Laboratories, Detroit, MI 48232.

⁵Bioscience Division of Milk Specialties Co., 3802 Packers Ave., Madison, WI 53704.

TABLE 1. Effect of dietary tannic acid (TA) on the concentrations of propionic acid and total volatile fatty acids in the cecal contents of broiler chicks at 3, 6, 8, and 11 d of age (Experiment 1)¹

Treatment ²	Propionic acid ($\mu\text{mol/g}$ cecal content)				Total volatile fatty acids ($\mu\text{mol/g}$ cecal content)			
	3 d	6 d	8 d	11 d	3 d	6 d	8 d	11 d
Control (NCEC)	1.96 ^a	0.37 ^a	6.83 ^a	6.10 ^a	38.57 ^a	24.88 ^a	59.86 ^a	91.45 ^a
0.75% TA (NCEC)	1.69 ^a	0.51 ^a	10.42 ^a	6.78 ^a	37.66 ^a	28.37 ^a	64.17 ^a	73.23 ^{ab}
1.50% TA (NCEC)	2.82 ^a	0.46 ^a	3.39 ^a	6.03 ^a	38.13 ^a	29.72 ^a	47.88 ^a	69.85 ^b
LSD ³	1.47	0.84	8.82	6.04	13.03	6.88	17.38	20.62
Control (CEC) ⁴	23.19 ^b	18.03 ^a	21.57 ^{ab}	38.78 ^b	53.46 ^{ab}	53.18 ^a	70.42 ^{ab}	148.78 ^b
0.75% TA (CEC)	30.66 ^a	21.14 ^a	25.34 ^a	65.77 ^a	64.48 ^a	56.90 ^a	84.87 ^a	183.48 ^a
1.50% TA (CEC)	17.80 ^b	17.93 ^a	16.91 ^b	27.92 ^b	42.55 ^b	51.31 ^a	55.32 ^b	99.86 ^c
LSD ⁴	7.08	6.38	7.74	15.85	14.82	14.13	16.14	32.48

^{a-c}Means within a column and culture treatment with different superscripts differ significantly ($P < 0.05$).

¹Values represent the mean of 12 chicks per treatment (four replicates of three chicks each) for each sampling.

²NCEC = noncompetitive exclusion (native microflora); CEC = competitive exclusion treated.

³LSD = least-significant difference as determined by Fisher's protected LSD procedure.

⁴Competitive exclusion culture = PREEMPT™ Bioscience Division of Milk Specialties Co., Madison, WI 53704.

at 11 d for propionic acid and total VFA, respectively. Within the groups of CEC-treated chicks compared with controls, concentrations of cecal propionic acid were significantly elevated at 3 and 11 d in chicks fed the 0.75% TA diet. Concentrations of total VFA were significantly increased at 11 d in chicks fed the 0.75% TA diet and were decreased in chicks fed the 1.5% TA diet. The range of values for the CEC treatments were 5.36 to 47.49 and 11.45 to 88.04 at 3 d, 10.14 to 32.81 and 33.23 to 88.27 at 6 d, 3.43 to 40.04 and 26.52 to 112.20 at 8 d, and 10.14 to 104.31 and 56.09 to 237.89 at 11 d for propionic acid and total VFA, respectively. When compared with chicks in the NCEC treated groups, there was a significant increase in cecal concentrations of propionic acid and total VFA in the chicks in the CEC-treated groups.

When compared with controls at the termination of the experiment, body weights were significantly reduced in chicks fed the 0.75% TA diet and further reduced in chicks fed the 1.5% TA diet in the NCEC and CEC groups of chicks (Table 2). Body weights ranged from 144 to 319 g. Within the NCEC and CEC treatments, there was no significant treatment difference observed for numbers

of *Salmonella* cecal culture-positive chicks or in numbers of ST in the cecal contents due to TA treatment. When compared with the chicks in the NCEC-treated group, there was a highly significant reduction in number of *Salmonella* cecal culture-positive chicks ($P < 0.01$) and in the number of ST in the cecal contents in the chicks for the CEC-treated groups. The range of values for log₁₀ *Salmonella* per gram of cecal contents was 0.00 to 7.30 for NCEC treatments and 0.00 to 5.11 for CEC treatments.

The data for concentrations of cecal propionic acid and total VFA in Experiment 2 are presented in Table 3. Within the groups of NCEC-treated chicks, when compared with NCEC controls, concentrations of propionic acid were decreased at 4 d in chicks fed the 1.5% TA diet and at 12 d in chicks fed the 0.75 and 1.5% TA diets. Within the groups of NCEC-treated chicks, there were no alterations in total VFA. The range of values for NCEC treatments was 0.00 to 4.66 and 10.24 to 42.03 at 4 d, 0.00 to 5.31 and 19.44 to 84.47 at 8 d, and 3.45 to 20.04 and 39.86 to 117.73 at 12 d for propionic acid and total VFA, respectively. Within the groups of CEC-treated chicks, concentrations of propionic acid were decreased

TABLE 2. Effect of dietary tannic acid (TA) on body weights, the number of chicks cecal culture-positive for *Salmonella typhimurium*, and the number of *Salmonella typhimurium* in the cecal contents of 11-d-old broiler chicks (Experiment 1)¹

Treatment ²	Body weights g	<i>Salmonella</i> culture-positive chicks/total (%)	Log ₁₀ <i>Salmonella</i> per g cecal contents (number of chicks)
Control (NCEC)	244 ^a	17/20 (85)	4.80 ^a (20)
0.75% TA (NCEC)	206 ^b	17/20 (85)	4.49 ^a (20)
1.5% TA (NCEC)	144 ^c	18/20 (90)	5.57 ^a (20)
		52/60 (87)	
Control (CEC)	231 ^a	2/20 (10)	0.17 ^b (20)
0.75% TA (CEC)	196 ^b	1/20 (5)	0.15 ^b (20)
1.50% TA (CEC)	144 ^c	4/20 (20)	0.78 ^b (20)
LSD ³	15	7/60 (12)**	1.60

^{a-c}Means within a column and culture treatment with different superscript differ significantly ($P < 0.05$).

¹Competitive exclusion (CE) culture = PREEMPT™ Bioscience Division of Milk Specialties Co., Madison, WI 53704.

²NCEC = noncompetitive exclusion (native microflora); CEC = competitive exclusion treated.

³LSD = Least-significant difference as determined by Fisher's protected LSD procedure.

**Significantly different from combined non-CE culture-treated chicks ($P < 0.01$).

TABLE 3. Effect of dietary tannic acid (TA) on the concentrations of propionic acid and total volatile fatty acids in the cecal contents of broiler chicks at 3, 6, 8, and 11 d of age (Experiment 2)¹

Treatment ²	Propionic acid ($\mu\text{mol/g}$ cecal content)			Total volatile fatty acids ($\mu\text{mol/g}$ cecal content)		
	4 d	8 d	12 d	4 d	8 d	12 d
Control (NCEC)	2.25 ^a	3.31 ^a	9.57 ^a	31.32 ^a	49.79 ^a	81.89 ^a
0.75% TA (NCEC)	0.96 ^{ab}	2.72 ^a	4.78 ^b	25.53 ^a	56.67 ^a	72.61 ^a
1.50% TA (NCEC)	0.32 ^b	2.30 ^a	5.22 ^b	26.17 ^a	42.75 ^a	69.79 ^a
LSD ³	1.42	1.77	3.08	6.11	15.56	17.29
Control (CEC) ⁴	7.88 ^a	7.29 ^a	16.14 ^a	31.70 ^a	54.63 ^a	102.06 ^a
0.75% TA (CEC)	6.96 ^{ab}	8.19 ^a	15.16 ^a	28.12 ^{ab}	46.14 ^a	90.60 ^a
1.50% TA (CEC)	5.48 ^b	5.92 ^a	13.43 ^a	24.94 ^b	34.49 ^b	88.31 ^a
LSD ⁴	2.10	2.48	6.24	6.16	12.19	23.13

^{a-c}Means within a column and culture treatment with different superscripts differ significantly ($P < 0.05$).

¹Values represent the mean of 12 chicks per treatment (four replicates of three chicks each) for each sampling.

²NCEC = noncompetitive exclusion (native microflora); CEC = competitive exclusion treated.

³LSD = Least-significant difference as determined by Fisher's protected LSD procedure.

⁴CE culture = PREEMPT™ Bioscience Division of Milk Specialties Co., Madison, WI 53704.

at 4 d in chicks fed the 1.5% TA diet. Within the groups of CEC chicks, total cecal VFA concentrations were significantly decreased at 4 d and 8 d in chicks fed the 1.5% TA diet. The range of values for CEC treatments was 3.59 to 12.39 and 17.00 to 42.98 at 4 d, 0.00 to 17.98 and 22.49 to 86.08 at 8 d, and 5.35 to 35.34 and 51.51 to 158.78 for propionic acid and total VFA, respectively. The cecal propionic acid concentrations were higher in chicks treated with the CE culture (CEC) than the untreated chicks (NCEC) at 4, 8, and 12 d. Total VFA were not increased in the CEC chicks, when compared with the NCEC chicks.

The experimental challenge dose of 10^4 cfu ST resulted in cecal colonization of 77% of the chicks in the NCEC groups. There were no differences due to TA treatment (Table 4). This challenge resulted in a significant ($P < 0.05$) decrease in cecal colonization to 42% of the chicks in the CEC groups. Compared with the chicks in the

NCEC-treated groups, the numbers of ST in the cecal contents of the chicks in the CEC-treated groups decreased ($P < 0.05$) by 3.22 \log_{10} units. The range of values for \log_{10} *Salmonella* per gram of cecal contents was 0.00 to 7.01 for NCEC treatment and 0.00 to 6.74 for CEC treatment. There were no significant differences in the numbers of ST in the cecal contents due to feeding diets containing TA.

DISCUSSION

When compared with controls, feeding young chicks 0.75% or 1.5% TA in the diet for 11 d resulted in reduced body weights of 16 and 41%, respectively. These results agree with the results of Chang and Fuller (1964), Vohra et al. (1966), Conner et al. (1969), Rostagno et al. (1973), Armstrong et al. (1973, 1974), Dale et al. (1980), and Kubena et al. (1983).

Propionic acid concentrations were consistently higher in the CEC-treated chicks than in the NCEC-treated chicks in both experiments. When looking at the overall data of the two experiments, there was a ninefold increase in the cecal concentration of propionic acid in the CE-treated chicks, compared with the non-CE-treated chicks at the first sampling time. Cecal concentrations of total VFA in the CEC-treated chicks were numerically, when not statistically, higher than the NCEC-treated chicks. The expected increase in propionic acid and total VFA concentrations detected in chicks administered the CE culture is a characteristic indication of the establishment of the culture in the digestive tract (Nisbet et al., 1994; Corrier et al., 1995; Droleskey et al., 1995). The reason for the lower concentrations of cecal propionic acid in Experiment 2, when compared with Experiment 1 and some other reports, is unknown. Perhaps in this second experiment, a different microbial population was present in the ceca before the chicks were administered the CE culture. When considering all treatments, the challenge dose of 10^4 cfu ST resulted in 75 to 90% of the non-CE-treated chicks being *Salmonella* cecal culture-positive; the \log_{10} *Salmonella* per gram of cecal contents

TABLE 4. Effects of tannic acid (TA) on the number of chicks cecal culture-positive for *Salmonella typhimurium* and the number of *S. typhimurium* in the cecal contents of 12-d-old broiler chicks (Experiment 2)¹

Treatment ²	<i>Salmonella</i> culture-positive chicks/total (%)	\log_{10} <i>Salmonella</i> per g cecal contents (number of chicks)
Control (NCEC)	15/20 (75)	4.74 ^a (20)
0.75% TA (NCEC)	15/20 (75)	4.25 ^a (20)
1.5% TA (NCEC)	16/20 (80)	5.01 ^a (20)
	46/60 (77)	
Control (CEC)	7/20 (35)	1.77 ^b (20)
0.75% TA (CEC)	9/20 (45)	1.70 ^b (20)
1.50% TA (CEC)	9/20 (45)	1.93 ^b (20)
LSD ²	25/60 (42)*	1.79

^{a,b}Means within a column with different superscript differ significantly ($P < 0.05$).

¹Competitive exclusion (CE) culture = PREEMPT™ Bioscience Division of Milk Specialties Co., Madison, WI 53704.

²NCEC = noncompetitive exclusion (native microflora); CEC = competitive exclusion treated.

³LSD = Least-significant difference as determined by Fisher's protected LSD procedure.

*Significantly different from combined non-CE culture-treated chicks ($P < 0.05$).

for the controls, 0.75% TA, and 1.5% TA were 4.77, 4.37, and 5.29, respectively. Treatment of the chicks at day of hatch with the CE culture resulted in a decrease in *Salmonella* cecal culture-positive chicks (27 vs. 82%) and a significant decrease in the number of *Salmonella* per gram of cecal contents for the controls to 0.97, for the 0.75% TA to 0.93, and for the 1.5% TA to 1.36. The significant decrease in propionic acid in the NCEC chicks at 1.5% TA at 4 d, and at 0.75% and 1.5% at 12 d, in Experiment 2 did not cause a change in susceptibility to ST. The significant decrease in propionic acid in the CEC chicks at 4 d and the decrease in total VFA at 4 and 8 d did not change susceptibility to ST. Interestingly, chicks with 5.48 μmol cecal propionic acid/g of cecal contents were protected to the same extent from cecal colonization by *Salmonella* as chicks with slightly higher concentrations of propionic acid (6.96 and 7.88). These results agree with the results of Nisbet et al. (1996) and Kubena et al. (2001). Nisbet et al. (1996) observed significant protection with 7.5 μmol propionic acid/g of cecal contents but did not observe protection at concentrations of 2.1 to 5.4. Kubena et al. (2001) showed protection at concentrations of propionic acid of 5.4 and 5.1 μmol per g of cecal contents. It is unlikely that the increases in propionic acid concentrations observed in these earlier studies are solely responsible for the decrease in colonization observed, but they could be due to other factors such as competition for limiting nutrients required for optimal propionic acid formation (Nisbet et al., 1996). Ha et al. (1994) demonstrated that competition for serine in an anaerobic environment was an important factor in a co-culture containing ST and a native cecal bacterial isolate. Although it is not well understood how propionic acid functions in the mechanisms of reduction of *Salmonella* cecal colonization, the present research supports the previous work of Corrier et al. (1995), Nisbet et al. (1994, 1996), and Kubena (2001), showing it is a biological indicator correlated with *Salmonella* control.

Results of the present research support previous research showing that the concentration of propionic acid produced in the ceca of young chicks may be an important part of the mechanisms that inhibit ST colonization of young chicks by anaerobic bacteria. These results also indicate that cecal concentrations of VFA can be affected by dietary constituents such as TA, but these alterations in VFA do not appear to be severe enough to affect *Salmonella* colonization. This research shows that dietary TA did not compromise the efficacy of a CE product against ST, even when included at levels high enough to depress growth rate.

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